Evolutionary Ecology Research, 2016, **17**: 1–16

Parallelism and divergence in immune responses: a comparison of expression levels in two lakes

Shaun Robertson, Janette E. Bradley and Andrew D.C. MacColl

School of Life Sciences, University of Nottingham, Nottingham, UK

ABSTRACT

Question: How do immune phenotypes differ between infected and uninfected wild individuals, and is the effect the same in different populations?

Organisms: Threespine stickleback (*Gasterosteus aculeatus*) from two lake populations on the island of North Uist, Scotland, sampled in May 2015.

Methods: For each fish, we recorded length, sex, reproductive status, condition, and parasitic infection. We measured the expression levels of eight genes that act as key markers of immune system function using qPCR, and then examined the relationship between measured factors and immune gene expression profiles within each population.

Conclusions: Populations differed significantly in their immune gene expression profiles. Within each population, multiple factors, including condition, reproductive status, and *Schistocephalus solidus* infection levels, were found to correlate with expression levels of different arms of the immune system.

Keywords: *Gasterosteus aculeatus*, gene expression, host–pathogen interactions, immunoecology, *Schistocephalus solidus*, threespine stickleback, wild immunology.

INTRODUCTION

Studies of the immune system to date have focused primarily on humans and a few select model species (Pedersen and Babayan, 2011; Maizels and Nussey, 2013). Such studies are typically laboratory-based and performed in highly controlled settings where variation is minimized, so that cellular and molecular mechanisms can be identified. This approach, however, is disconnected from the natural world where 'real' immune systems have to operate (Pedersen and Babayan, 2011), where there is genetic diversity of both hosts and parasites, and variation in the abiotic environment. Such diversity may play an important role in shaping the function of the immune system in the wild (Lazzaro and Little, 2009; Martin *et al.*, 2011), and placing current knowledge of immunological mechanisms into this real-world context is a fundamentally important challenge in advancing our understanding of the immune system from an evolutionary perspective (Maizels and Nussey, 2013).

Correspondence: S. Robertson, School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK. e-mail: plxsr3@nottingham.ac.uk

The expansion of the study of the immune system in the wild is constrained by a lack of appropriate assays and study species (Fassbinder-Orth, 2014), and studies applying modern immunological measurements to natural populations by simultaneously measuring multiple markers of the immune system are rare and primarily limited to rodents (Oko *et al.*, 2006; Schountz *et al.*, 2007; Jackson *et al.*, 2014). Expression assays represent an obvious starting point for the study of the immune response in organisms that lack reagents (particularly for protein-level analysis), although results can be difficult to interpret and data from wild populations inherently noisy. One way to address the possible limitations of such an approach is to compare expression profiles between different populations in relation to candidate causal factors, such as parasitic infection levels, sex or size. If signals are consistent across locations, we can have more confidence that the patterns identified are 'real'. Due to the restricted set of previous studies, there is a limited understanding of the role of an individual's environment in shaping immune function in a natural setting; the threespine stickleback, *Gasterosteus aculeatus* L. (hereafter 'stickleback'), is ideally suited to address this issue.

Sticklebacks are a commonly studied model species in evolution and ecology (see, for example, Hendry *et al.*, 2013). Populations of stickleback show stable differences in parasite community composition (Scharsack *et al.*, 2007a; De Roij and MacColl, 2012), and interactions with a range of parasites have been well documented (Kalbe and Kurtz, 2006; MacColl, 2009; Barber, 2013; Konijnendijk *et al.*, 2013). There is growing evidence for within- and between-population variation in parasite resistance (De Roij *et al.*, 2011), which probably has a genetic basis (Rauch *et al.*, 2006; El Nagar, 2014). This long history of study, combined with the ability to sample replicate populations in the wild and perform controlled infection experiments in the laboratory, has made the stickleback an excellent model in the study of evolutionary and ecological parasitology (Barber, 2013), and an ideal system in which to conduct immunological studies.

The stickleback found on the island of North Uist, Scotland, represent one well-studied system (Giles, 1983; De Roij and MacColl, 2012; MacColl *et al.*, 2013). A number of parasite species infect sticklebacks on North Uist, including *Gyrodactylus arcuatus*, an ectoparasitic trematode, and *Schistocephalus solidus*, a pseudophyllidean cestode. Infections with *S. solidus* can have a negative impact on host fitness (Barber *et al.*, 2008; Barber and Scharsack, 2010), and parasites have been shown to modulate the immune response of the host (Barber and Scharsack, 2010). Although the impact of infection on host fitness of a number of parasites has been well studied, the link between infection and a host's defence is less clear.

Previous research has examined the immune response of sticklebacks, particularly the role of immune-related cell populations (Scharsack *et al.*, 2004) and the major histocompatibility complex (MHC) (Kurtz *et al.*, 2004; Wegner *et al.*, 2006). The genetic background of an individual appears to play a role in determining parasite load (Rauch *et al.*, 2006; Eizaguirre *et al.*, 2011), suggesting adaptation to local parasite populations, which could be modulated through the immune response.

Recent advances allow the expression levels of immune-related genes to be measured directly. Next-generation sequencing of the transcriptome of sticklebacks has shown adaptation of expression levels of immune genes to local, co-evolved, parasite strains (Lenz *et al.*, 2013), and changes in gene expression that are dependent upon the genotype of the infecting parasite (Haase *et al.*, 2014) in controlled, laboratory-based, infection experiments. This whole-transcriptome sequencing approach has provided new insights into the response of individuals to controlled infections, but there is a relatively high cost associated with sequencing the large numbers of biological replicates required for such studies of wild populations. An alternative approach to whole-transcriptome sequencing is to use

quantitative real-time PCR (qPCR) to measure the expression of pre-selected genes (Fassbinder-Orth, 2014), chosen based on *a priori* knowledge of the function of the immune system. Such studies have proved fruitful in the study of rodents (Jackson *et al.*, 2009, 2011), and qPCR has successfully been applied to stickleback (Hibbeler *et al.*, 2008; Dittmar *et al.*, 2014; Robertson *et al.*, 2015).

To provide a cohesive overview of the function of the immune system, we developed a set of qPCR assays that measure the innate response, the Th1-type and Th2-type adaptive responses, and the regulatory response (Robertson *et al.*, 2015). In this study, we employ these assays to measure the immune response of wild stickleback from two lake populations on the island of North Uist, Scotland. We selected populations that typically have similar high rates of infection with *S. solidus* (A.D.C. MacColl, unpublished data), but are geographically isolated from each other. In this way, we hoped to detect changes in immune gene expression levels that were associated with *S. solidus* infection, above the natural variation expected in data from wild individuals. Furthermore, we examined which factors relate to immune gene expression levels within each population and looked at whether common factors are involved in shaping the immune response in the wild. This research adds to the growing use of stickleback as an alternative species in the study of the immune system, and begins to examine which factors play a role in shaping immune gene expression in a natural setting.

MATERIALS AND METHODS

Sample collection

All work involving animals was approved by the University of Nottingham ethics committee, under UK Home Office licence (PPL-40/3486), and sampling on North Uist was conducted with the permission of North Uist Estates. Fish were sampled from Loch a'Bharpa ('Bhar', 57°34′20″N, 7°18′11″W) and Loch Hosta ('Host', 57°37′30″N, 7°29′8″W) on the island of North Uist, Scotland, in May 2015. Forty-six fish were sampled from each population, split between two sampling days per population. Fish were caught in Gee's Minnow Traps, set overnight for approximately 16 hours. All individuals caught in a trapping session were pooled, and a subset was selected for inclusion in this study. Fish were transported directly to the laboratory in darkened conditions and processed in a haphazard order within 4 hours of collection. All fish were collected and processed before midday. No correlations were found between sampling order or sampling day and gene expression levels, indicating that our sampling procedure had no effect on expression levels.

Fish were euthanized using the appropriate schedule 1 technique according to UK Home Office regulations, then measured and weighed. Whole spleens, an immunologically important tissue in fish (Zapata *et al.*, 2006), were removed, weighed, and placed into RNAlater (Life Technologies). Sex was recorded along with reproductive status, determined by visual examination of the gonads. Male and female reproductive status was split into two categories: individuals with no apparent investment in reproduction (small gonads and kidneys in males, small ovules only in females), versus individuals investing in reproduction or ready to breed (gonads and/or kidneys showing enlargement in males, some or all ovules enlarged in females). The liver was removed and weighed, as was any adipose tissue in the body cavity.

Parasites were identified and counted under a dissection microscope. Where individuals were infected with *S. solidus*, the total weight of all plerocercoids (the life stage infecting

stickleback) was recorded, and the parasite index calculated as the total proportion of a fish's body weight accounted for by the parasite (Arme and Owen, 1967). The relative weights of the spleen (spleen somatic index, SSI), liver (hepato-somatic index, HSI), and adipose tissue (adipose index, AI) were calculated. Individual condition scores were calculated using Fulton's condition factor, $K = [10^5 \times \text{weight (g)}]/\text{length (mm)}^3$ (cited in Nash *et al.*, 2006), adjusted for individuals infected with *S. solidus* by using individual weight minus the total weight of all plerocercoids.

Sample preparation

All qPCR work was performed in accordance with the MIQE guidelines (Bustin *et al.*, 2009; Taylor *et al.*, 2010). Spleens were stored in RNAlater (Life Technologies) at −20°C for no longer than 3 months. RNA was extracted using the GeneJET RNA purification kit (Thermo Scientific) according to the manufacturer's standard protocol. Purity of RNA samples was assessed on a NanoDrop 1000 spectrophotometer (Thermo Scientific), with a desired 260/280 absorbance ratio > 1.80. Integrity of RNA was assessed by incubating 5 μL of sample at 65°C for 10 minutes, followed by visualization on a 2% agarose gel stained with ethidium bromide.

All samples were DNase treated using Precision DNase (Primer Design), following the manufacturer's protocol. Reverse transcription reactions were performed on approximately 1.5 μg of total RNA using the nanoScript2 RT kit (Primer Design), with a combination of random nanomer and oligo-dT priming, following the manufacturer's standard protocol. Periodic no-enzyme controls indicated that genomic DNA contamination was negligible. cDNA samples were diluted 1:10 with nuclease-free water before further use.

Gene expression quantification

All qPCR reactions were performed in 10 μL total volumes, containing 5 μL of PrecisionFAST mastermix with SYBR green (Primer Design), 0.25 μL of each primer, 2 μL of template cDNA, and 2.5 μL of H_2O in 96-well optical PCR plates with optical seals (StarLab). Reactions were performed in an ABI 7500 FAST real-time thermocycler (Applied Biosystems) at 95°C for 20 s, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. All runs included a post-PCR melt curve analysis.

Accurate normalization of gene expression is essential for the production of reliable data in qPCR experiments, with the optimal reference genes being specific to a particular set of experimental conditions (Dheda *et al.*, 2005). To select the most appropriate normalization strategy, a geNorm analysis was performed with six candidate reference genes (B2M, GAPDH, RPL13A, HPRT1, TBP, and TOP1) on 12 cDNA samples, randomly selected from all experimental samples, using a custom stickleback geNorm kit for SYBR green (Primer Design), following the manufacturer's standard protocol. Analysis of the stability of expression was performed in qbase + (Biogazelle) with B2M and RPL13A identified as the most stable combination of reference genes for this study.

Expression levels of eight genes of interest were measured, along with two reference genes. Genes of interest were IL-1β, TNFα, Stat4, Tbet, Stat6, CMIP, FoxP3, and TGFβ (for full details, see Robertson *et al.*, 2015). We made a reference sample by pooling cDNA from all experimental samples, to control for between-plate variation. A total of 92 cDNA samples

were split randomly between two plates, with reactions performed in duplicate for each sample, and each plate also contained the reference sample and negative controls.

Relative expression values were calculated using the ΔΔCq method (Pfaffl, 2001), adjusted for the amplification efficiencies of each primer pair and standardized against the geometric mean Cq of the two reference genes for each sample (Vandesompele *et al.*, 2002).

Data analyses

All expression values were $\log_{10}(x + 1)$ transformed prior to analysis, due to the inherently skewed distribution of relative expression data. Analysis was performed in R v.3.2.2 (R Core Development Team, 2014). We first examined whether any factors relate to variation in individual condition. Next, we investigated whether any factors related to immune gene expression variation in each population, by using principal components analysis (PCA) to summarize immune gene expression levels, then fitted general linear models containing recorded factors that we expected to influence expression.

Variation in individual condition

We summarized individual condition by using PCA on K, HSI, SSI, and AI, based on the correlation matrix. Principal components analysis was performed on both populations combined to ensure that each condition principal component (PC) was summarizing the same variation in the two populations, to allow direct comparisons of the relationships between condition measures and immune gene expression levels in the models fitted to Host and Bhar. Principal components were retained for use in further analysis if they had a standard deviation ≥1 and explained ≥10% of variance in the data. To examine which factors relate to the condition measures, general linear models were fitted separately to each population, with condition PC1 or PC2 as the response variable. Sex (2 levels), reproductive status (2 levels), *G. arcuatus* presence (2 levels), and *S. solidus* presence (2 levels) were included as factors, and length as a continuous variable, along with a sex × reproductive status interaction term. Non-significant terms were sequentially dropped from the full model to give a minimum adequate model, with the significance of the remaining components determined by Wald *F*-tests.

Summarizing immune expression

Profiles of the eight immune response genes were compared between the two lakes (Host and Bhar) using multivariate analysis of variance (MANOVA). Overall differences were assessed using the Pillai method, followed by examination of each immune type separately.

The expression of groups of functionally related components of the immune system was summarized using PCA, a widely used dimension reduction technique that converts a number of variables into principal components to summarize the variation present in the original measures. In this way, we can summarize the expression of the genes from each arm of the immune response in a single measure encompassing the majority of variation present in the original data. Genes were split into four functional groups based on their role: IL-1β and TNFα (innate response); Stat4 and Tbet (Th1-type adaptive response); Stat6 and CMIP (Th2-type adaptive response); and FoxP3 and TGFβ (regulatory response). Principal components analysis was performed separately on each immune group in each population using the 'prcomp' function, based on the variance–covariance matrix. In most cases, except Th2-type expression in Bhar, the dominant first principal component (PC1) explained

a high proportion of variation in the data (>69%), with both genes having positive loadings. Individual PC1 scores were used for subsequent analysis, except for the Th2-type response where Stat6 and CMIP relative expression values were fitted instead.

Factors relating to immune expression

General linear models were fitted to the two populations separately using each immune grouping PC1 score, or Stat6 and CMIP relative expression, as the response variable, to examine which factors related to expression levels. Maximal models were fitted with sex (2 levels), reproductive status (2 levels), *G. arcuatus* presence (2 levels), and *S. solidus* parasite index (2 levels) as factors, and condition PC1 and PC2 scores and length as continuous variables. Biologically relevant interaction terms were also fitted. Non-significant terms were sequentially dropped from the full model to give a minimum adequate model. The significance of remaining components was determined using Wald *F*-tests.

RESULTS

Schistocephalus solidus was found to be the dominant parasite species in both populations, with a prevalence of 54.3% (95% binomial confidence interval: 39.0–69.0%) and an average intensity in infected individuals of 8.6 ± 1.7 (±standard error) in Bhar, and a prevalence of 43.5% (95% binomial confidence interval: 28.9–58.9%) and intensity of 4.5 ± 1.0 in Host. *Gyrodactylus arcuatus* was also found, with a prevalence of 6.5% (95% binomial confidence interval: 1.4–10.8%) and an intensity on infected individuals of 2.0 ± 0.6 in Bhar, and a prevalence of 19.6% (95% binomial confidence interval: 9.4–33.9%) and an average intensity of 2.3 ± 0.3 in Host. No other parasite species were identified in the sampled fish at sufficient rates to include in the analysis.

Variation in individual condition

Principal components analysis was used to summarize the four different measures that reflect individual condition. Condition PC1 accounted for 41% of variation, with loadings of K = 0.134, HSI = 0.641, SSI = −0.489, and AI = −0.577, while PC2 accounted for 24.9% of variance, with loadings of K = 0.973, HSI = −0.119, SSI = 0.186, and AI = −0.062 (Fig. 1). Factors relating to condition PC1 and PC2 scores are summarized in Table 1. In both Bhar and Host, individuals investing in attaining reproductive condition had higher PC1 scores (Bhar: $F_{2,39} = 4.69$, $P = 0.036$; Host: $F_{2,39} = 12.08$, $P < 0.001$), and this also varied with sex (Bhar: $F_{2,39} = 4.89$, $P = 0.033$; Host: $F_{2,39} = 10.53$, $P < 0.001$) such that females had higher condition PC1 scores than males for a given reproductive condition score. Infection with *S. solidus* was associated with a decrease in condition PC1 score (Fig. 1) in both Bhar ($F_{1,39} = 37.57$, $P < 0.001$) and Host ($F_{1,39} = 10.594$, $P = 0.002$). In both Bhar and Host, larger fish had lower condition PC2 scores (Bhar: $F_{1,44} = 6.86$, $P = 0.012$; Host: $F_{1,40} = 13.51$, $P < 0.001$). In Host, males had lower condition PC2 scores than females ($F_{1,40} = 6.49$, $P = 0.015$), scores were higher in fish in reproductive condition ($F_{2,40} = 6.09$, $P = 0.005$), and infection with *S. solidus* was associated with an increase in condition PC2 score ($F_{1,40} = 5.01$, $P = 0.031$).

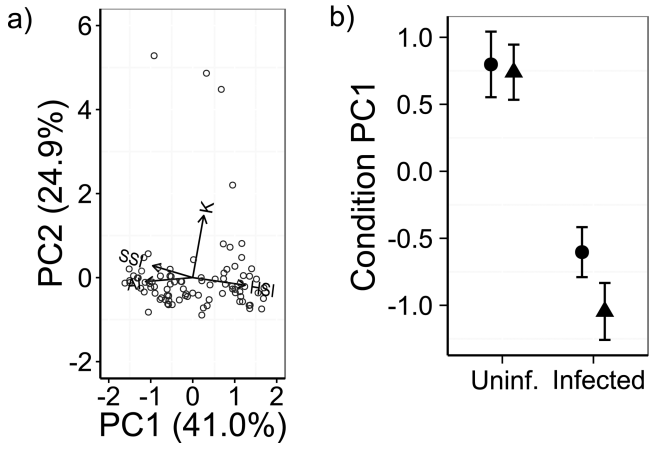

Fig. 1. (a) Individual scores of the first and second principal components (PC1 and PC2) of condition measures (with percent variation explained) on fish from two lake populations (Bhar and Host) on North Uist, Scotland. PC1 and PC2 summarize variation in Fulton's condition factor (*K*), adipose index (AI), spleen somatic index (SSI), and hepato-somatic index (HSI). (b) Fish infected with *S. solidus* ('Infected') had lower condition PC1 scores than uninfected fish ('Uninf.') in both study populations (● = Bhar, ▲ = Host). A high PC1 indicates a high hepato-somatic index, with a low spleen somatic index and adipose index.

Table 1. Summaries of minimum adequate models fitted to condition data from wild fish from two lakes ('Bhar' and 'Host') on North Uist, Scotland

	Bhar			Host		
Factor	*F*	d.f.	*P*	*F*	d.f.	*P*
Condition PC1						
Sex	4.50	1,39	0.040	0.05	1,39	0.826
Reproduction	4.69	1,39	0.036	12.08	1,39	<0.001
S. solidus presence	37.57	1,39	<0.001	10.59	1,39	<0.001
Sex × Reproduction	4.89	1,39	0.033	10.53	1,39	<0.001
Condition PC2						
Length	6.86	1,44	0.012	13.51	1,40	<0.001
Sex		n/a		6.49	1,40	0.005
Reproduction		n/a		6.09	1,40	0.015
S. solidus presence		n/a		5.01	1,40	0.031

Note: Condition PC1 represents variation in the hepato-somatic index (HSI), spleen somatic index (SSI). and adipose index (AI), while condition PC2 represents variation in Fulton's condition factor (*K*). Terms that were not included in the final minimum model for a population, but which were retained in the minimum model for the other population, are marked 'n/a'.

Summarizing immune expression

Fish from Bhar and Host differed significantly in their overall immune profiles (MANOVA: $F_{1,90} = 10.169$, $P < 0.001$) (Fig. 2). The populations differed in expression of TNFα ($F_{1,90} = 13.58$, $P < 0.001$), Stat6 ($F_{1,90} = 10.29$, $P = 0.002$), CMIP ($F_{1,90} = 14.30$, $P < 0.001$),

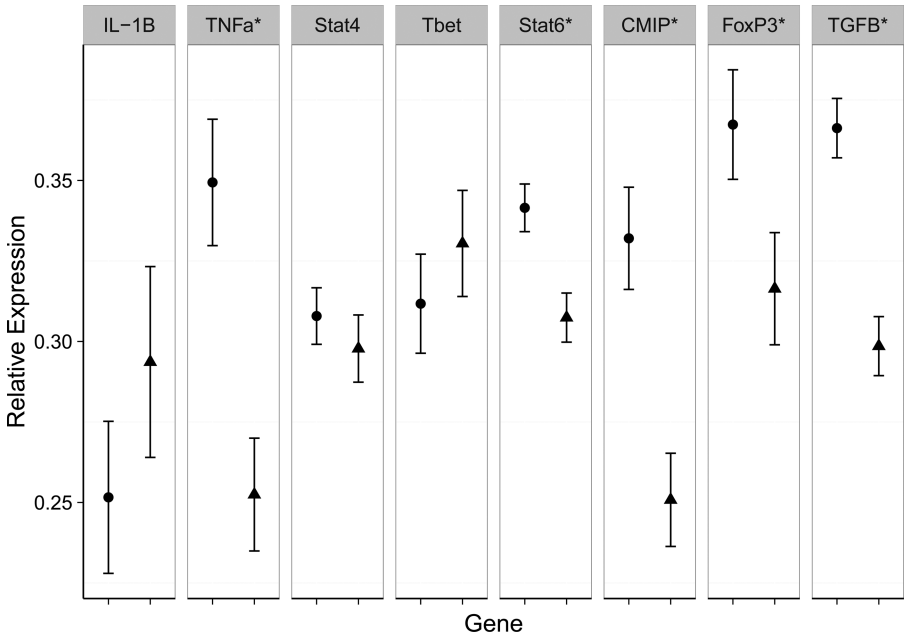

Fig. 2. Relative gene expression levels (mean ± standard error) of fish from two lake populations (● = Bhar, ▲ = Host) on North Uist, Scotland. Genes with significant expression differences are indicated with (*), with expression being lower in Host in all cases. IL-1β and TNFα represent the innate immune response, Stat4 and Tbet the Th1-type adaptive response, Stat6 and CMIP the Th2-type adaptive response, and FoxP3 and TGFβ the regulatory response.

FoxP3 ($F_{1,90} = 1.38$, $P = 0.039$), and TGFβ ($F_{1,90} = 27.13$, $P < 0.001$), but not IL-1β ($F_{1,90} = 1.23$, $P = 0.270$), Stat4 ($F_{1,90} = 0.55$, $P = 0.461$) or Tbet ($F_{1,90} = 0.69$, $P = 0.409$).

Grouped immune gene expression measures were summarized in each population using PCA, with high PC1 values in all groups indicating high expression of included genes. In Bhar, PC1 of innate immune measures explained 69.6% of variation in the data, with loadings of IL-1$\beta = 0.856$ and TNF$\alpha = 0.516$. For the Th1-type measures, PC1 explained 79.7% of variation, with loadings of Stat4 = 0.267 and Tbet = 0.964. The Th2-type measures were found to give PC1 loadings in opposite directions, so were kept separate for subsequent analysis. The regulatory measures gave a PC1 explaining 81.7% of variation, with loadings of FoxP3 = 0.965 and TGF$\beta = 0.263$.

In Host, PC1 of innate measures explained 78.5% of variation, with loadings of IL-1$\beta = 0.961$ and TNF$\alpha = 0.277$. For the Th1-type measures, PC1 explained 84.8% of variation, with loadings of Stat4 = 0.439 and Tbet = 0.898. For the Th2-type measures, PC1 explained 81.4% of variation, with loadings of Stat6 = 0.222 and CMIP = 0.975. The regulatory measures PC1 accounted for 82.2% of variation, with loadings of FoxP3 = 0.969 and TGF$\beta = 0.245$.

Factors relating to immune expression

A range of factors was found to relate to expression levels of different arms of the immune system, as summarized in Table 2. Some factors relating to innate and Th1-type expression

Table 2. Summaries of minimum adequate models fitted to expression data from wild fish from two lakes ('Bhar' and 'Host') on North Uist, Scotland

	Bhar				Host			
Factor	*F*	d.f.	*P*	Effect	*F*	d.f.	*P*	Effect
Innate								
Length	14.11	1,40	**<0.001**	–	8.20	1,38	**0.007**	–
Sex		n/a			4.84	1,38	**0.034**	–
Condition PC1		n/a			12.94	1,38	**<0.001**	+
Condition PC2	5.48	1,40	**0.024**	+	11.32	1,38	**0.002**	–
G. arcuatus presence		n/a			5.19	1,38	**0.028**	+
S. solidus presence	0.39	1,40	0.534	–	6.59	1,38	**0.014**	+
Length × Sex		n/a			5.84	1,38	**0.021**	+
Length × Condition PC2	5.30	1,40	**0.027**	–		n/a		
Condition PC2 × *S. solidus* presence	6.85	1,40	**0.012**	–		n/a		
Th1								
Length		n/a			3.89	1,39	**0.056**	–
Sex	10.59	1,37	**0.002**	–		n/a		
Reproduction	0.14	1,37	0.714	–	0.46	1,39	0.540	–
Condition PC1	9.03	1,37	**0.005**	–	3.69	1,39	0.062	–
Condition PC2	0.15	1,37	0.697	+		n/a		
S. solidus presence	13.51	1,37	**<0.001**	–	11.07	1,39	**0.002**	–
Length × *S. solidus* presence		n/a			5.70	1,39	**0.022**	+
Sex × Reproduction	5.90	1,37	**0.020**	+		n/a		
Sex × Condition PC2	7.71	1,37	**0.009**	–		n/a		
Reproduction × Condition PC1	8.87	1,37	**0.005**	+	5.15	1,39	**0.029**	+
Stat6								
Length	0.52	1,36	0.476	+	7.61	1,40	**0.009**	+
Sex	4.77	1,36	**0.036**	–		n/a		
Reproduction		n/a			11.87	1,40	**0.001**	+
Condition PC1	3.42	1,36	0.072	+	2.60	1,40	0.115	–
Condition PC2	0.07	1,36	0.793	+		n/a		
S. solidus presence	0.87	1,36	0.358	+		n/a		
Length × : Reproduction		n/a			14.28	1,40	**<0.001**	–
Length × Condition PC1	4.43	1,36	**0.042**	–		n/a		
Sex × Condition PC1	8.35	1,36	**0.007**	–		n/a		
Sex × Condition PC2	4.91	1,36	**0.033**	–		n/a		
Reproduction × Condition PC1		n/a			5.95	1,40	**0.019**	+
Condition PC1 × *S. solidus* presence	6.31	1,36	**0.017**	+		n/a		
Regulatory								
Length	2.29	1,38	0.139	+	6.20	1,43	**0.017**	–
Condition PC1	2.53	1,38	0.120	–		n/a		
Condition PC2	3.79	1,38	0.060	+	6.06	1,43	**0.018**	–
S. solidus presence	6.33	1,38	**0.016**	+		n/a		
Length × *S. solidus* presence	5.91	1,38	**0.020**	–		n/a		
Condition PC1 × *S. solidus* presence	6.33	1,38	**0.016**	+		n/a		
Condition PC2 × *S. solidus* presence	7.11	1,38	**0.011**	–		n/a		

Note: 'Innate', 'Th1', and 'Regulatory' response variables are first principal components of the following pairs of genes respectively: IL-1β and TNFα; Stat4 and Tbet; FoxP3 and TGFβ. Significant *P*-values ($P < 0.05$) are marked in **bold**. The direction of the effect (+ or –) of each factor or interaction term is shown (male relative to female for 'Sex', reproductive relative to non-reproductive for 'Reproduction'). Terms that were not included in the final minimum model for a population, but which were retained in the minimum model for the other population, are markedn/a'.

levels had the same effects in the two populations, while others either had opposing effects or were only related to expression levels within a single population.

Innate expression

In Bhar, larger fish had lower innate expression levels ($F_{1,40} = 14.11$, $P < 0.001$) (Fig. 3). An increase in condition PC2 was associated with an increase in innate expression levels ($F_{1,40} = 5.48$, $P = 0.024$), with the effect being less pronounced as fish got bigger ($F_{1,40} = 5.30$, $P = 0.027$). Individuals infected with *S. solidus* had lower innate expression levels at a given condition PC2 score ($F_{1,40} = 6.85$, $P = 0.012$). Larger fish in Host also had lower innate expression levels ($F_{1,38} = 8.20$, $P = 0.007$) (Fig. 3), but this also varied by sex ($F_{1,38} = 5.84$, $P = 0.021$), with males showing a larger decrease in expression with increasing size. An increase in condition PC1 score was associated with an increase in innate expression ($F_{1,38} = 12.94$, $P < 0.001$), while an increase in condition PC2 was associated with a decrease ($F_{1,38} = 11.32$, $P = 0.002$). In contrast to Bhar, infection with both *G. arcuatus* ($F_{1,38} = 5.19$, $P = 0.028$) and *S. solidus* ($F_{1,38} = 6.59$, $P = 0.014$) was associated with an increase in innate expression levels.

Th1-type expression

In Host, fish infected with *S. solidus* had lower Th1-type expression levels ($F_{1,39} = 11.07$, $P = 0.002$) (Fig. 4), with the difference increasing as fish got larger ($F_{1,39} = 5.70$, $P = 0.022$). Individuals in reproductive condition had higher Th1-type expression levels for a given

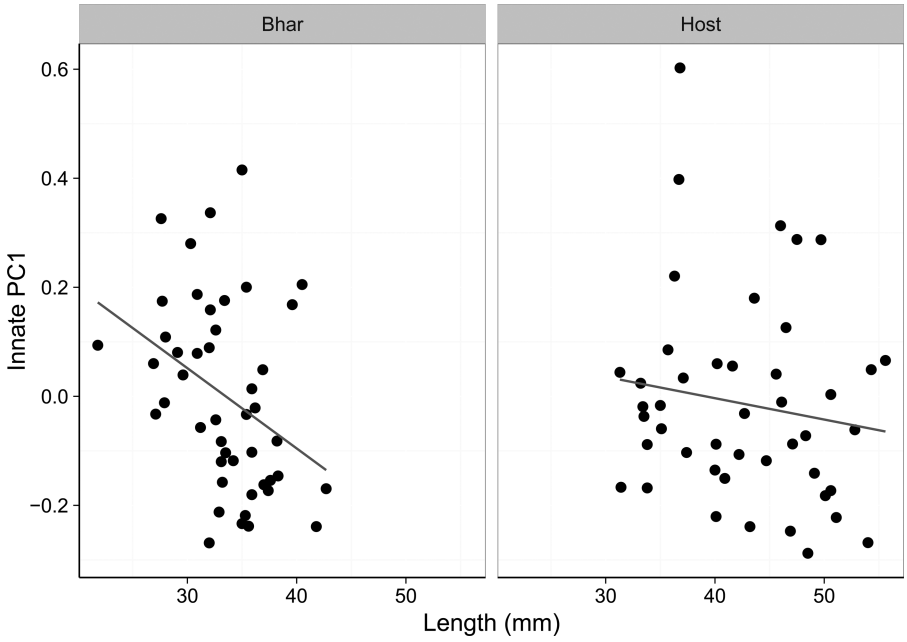

Fig. 3. Larger fish had lower innate gene expression levels (mean ± standard error) in two lake populations (Bhar and Host) on North Uist, Scotland (including linear best fit lines). The innate response variable is the first principal component scores of IL-1β and TNFα gene expression levels, with PCA performed separately on fish from each population. A high PC1 score indicates high expression levels of both genes.

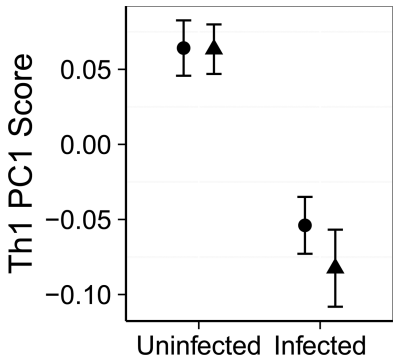

Fig. 4. Fish infected with *S. solidus* had lower Th1-type gene expression levels (mean ± standard error) in two lake populations (● = Bhar, ▲ = Host) on North Uist, Scotland. The Th1 response variable is the first principal component scores of Stat4 and Tbet gene expression levels, with PCA performed separately on fish from each population. A high PC1 score indicates high expression levels of both genes.

condition PC1 score ($F_{1,39} = 5.15$, $P = 0.029$). In Bhar, fish infected with *S. solidus* also had lower Th1-type expression than uninfected fish ($F_{1,37} = 13.51$, $P < 0.001$) (Fig. 4). Males had lower expression levels than females ($F_{1,37} = 10.59$, $P = 0.002$), and this effect varied with reproductive status ($F_{1,37} = 5.90$, $P = 0.020$), with the difference between the sexes much greater in non-reproductive individuals. Sex also altered the relationship between condition PC2 score and Th1-type expression ($F_{1,37} = 7.71$, $P = 0.009$), with the difference between the sexes decreasing as condition PC2 score increased. As in Host, the effect of condition PC1 score also varied with reproductive status in Bhar ($F_{1,37} = 8.87$, $P = 0.005$), with reproductive individuals having lower Th1-type expression at a given condition PC1 score.

Th2-type expression

No significant factors were found in the models for CMIP expression in Bhar or Host. In Bhar, males showed lower Stat6 expression levels than females ($F_{1,36} = 4.77$, $P = 0.036$). Sex influenced the relationship between condition PC1 and Stat6 expression ($F_{1,36} = 8.35$, $P = 0.007$) and condition PC2 and Stat6 expression ($F_{1,36} = 4.91$, $P = 0.033$), with males having lower expression than females for a given condition PC score. The effect of condition PC1 score on Stat6 expression also varied with length ($F_{1,36} = 4.43$, $P = 0.042$), with larger fish having lower Stat6 expression levels at a given condition PC1 score. The effect of condition PC1 score on Stat6 expression also varied with *S. solidus* infection ($F_{1,36} = 6.31$, $P = 0.017$), with infected fish having higher Stat6 expression levels than uninfected fish at a given condition PC1 score. In Host, larger individuals had higher Stat6 expression levels ($F_{1,40} = 7.61$, $P = 0.009$), as did individuals in reproductive condition ($F_{1,40} = 11.87$, $P = 0.001$). The effect of length also varied with reproduction ($F_{1,40} = 14.28$, $P < 0.001$), with the difference between reproductive and non-reproductive individuals decreasing with increasing length. Individuals in reproductive condition had higher Stat6 expression levels than non-reproductive individuals at a given condition PC1 score ($F_{1,40} = 5.95$, $P = 0.019$).

Regulatory expression

In Host, larger fish had lower regulatory gene expression levels ($F_{1,43} = 6.20$, $P = 0.017$), while an increase in condition PC2 score was associated with a decrease in expression levels

($F_{1,43} = 6.06$, $P = 0.018$). In Bhar, individuals infected with *S. solidus* had higher regulatory gene expression levels than uninfected individuals ($F_{1,33} = 6.33$, $P = 0.016$), but this difference decreased with increasing fish length ($F_{1,33} = 5.91$, $P = 0.020$). The difference in regulatory gene expression levels between *S. solidus* infected and uninfected individuals decreased with increasing condition PC1 score ($F_{1,33} = 6.33$, $P = 0.016$), and with increasing condition PC2 score ($F_{1,33} = 7.11$, $P = 0.011$).

DISCUSSION

In the present study, we observed differences in immune gene expression levels between populations, and identified a range of factors that contribute to shaping this expression. Data collected from wild populations is inherently noisy, yet we were able to detect variation in immune gene expression levels that are associated with infection with two common parasite species, *Schistocephalus solidus* and *Gyrodactylus arcuatus,* as well as associations with a range of other factors. Overall gene expression profiles differed between fish from Host and Bhar, primarily driven by higher expression levels of Th2-type and regulatory genes in Bhar. Previous work has shown that underlying gene expression levels differ between populations (Robertson *et al.*, 2015), and an individual's ability to respond has some genetic basis (Rauch *et al.*, 2006; Robertson *et al.*, 2015), thus the measured variation in expression here could represent underlying differences in responsiveness between populations, or differences in the challenges being faced in each population.

A range of factors were found to contribute to shaping expression of each response type, with factors relating to different arms of the immune system in different ways. Some explanatory factors were found to have common affects in both populations, including size, sex, and condition, while others had opposing affects or only related to expression levels in a single population. Interactions between the factors also varied. Recent thinking in immunology suggests that controlled laboratory-based studies of the immune system lack complexity compared with natural settings (Pedersen and Babayan, 2011; Bradley, 2015). The broad set of factors found here which relate to gene expression levels, and the interactions between them, confirm that immune function in the wild is complex, and shows that studies in immunology should consider multiple factors simultaneously.

Both sex and reproductive status correlated with an individual's immune expression profile. Previous studies in vertebrates have found that there are general differences in immune function between the sexes (Restif and Amos, 2010; Hawley and Altizer, 2011), and we expected males to have lower expression levels in general than females. Sex correlated with innate expression levels in Host, and with Th1-type, Th2-type, and Stat6 expression levels in Bhar; in all these cases, males had lower expression levels than females. Investing in reproduction can also influence immune response ability (Downs *et al.*, 2014). Individuals investing in reproduction had lower Th1-type gene expression levels in both Bhar and Host, and had higher Stat6 expression levels in Host. During the breeding season, the immune response can be affected by circulating hormone levels (Maule *et al.*, 1996; Cuesta *et al.*, 2007), and other factors, such as sex-specific behavioural changes, may also play a role. Further studies at different times of the year could indicate whether such sex differences are transient, and longitudinal studies could show whether different investment strategies affect breeding success, and ultimately fitness.

Infection with *S. solidus* was associated with variation in expression of all immune response types, but some of these effects differed between the studied populations. The

immune response of individuals has been shown to change with *S. solidus* infection in experimental studies (Scharsack *et al.*, 2004, 2007b), with growing evidence of parasite-driven manipulation of the host immune system (for a review, see Barber and Scharsack, 2010). The role of Th1/Th2 differentiation during *S. solidus* infection is not well understood due to a previous lack of appropriate assays. In this study, we saw a decrease in Th1-type expression with infection in both populations, as previously observed in wild populations on North Uist (Robertson *et al.*, 2015), and an increase in Th2-type and regulatory expression in Bhar only. Work in mice on Schistosomes, a digenean trematode residing in the bloodstream, has shown a shift towards an increased Th1-type response in order to avoid the potentially harmful Th2 or humoral type responses (Herve *et al.*, 2003), an approach that may be employed by *S. solidus* (Barber and Scharsack, 2010). Our data, however, show the opposite change in Th1-type response. Our results support the involvement of the adaptive response during infection, but a better understanding of the Th1/Th2 mechanism in teleost fish is required to shed light on the role of *S. solidus* in manipulating the host's immune system.

Condition measures were found to relate to expression levels of all response types in both populations, although the effect and interactions of the condition factors varied between populations. The condition PC1 axis represents opposing changes in liver size against spleen size and adipose fat tissue, and was associated with differences between the sexes, in reproductive condition, and *S. solidus* infection levels. Changes in liver, spleen, and adipose tissue were expected to correspond with *S. solidus* infection (Arme and Owen, 1967). The condition PC2 axis represents general body condition, measured as Fulton's condition factor, and generally decreased with increasing length. In turn, the condition PC1 and PC2 scores interacted with a number of factors in the models for both Bhar and Host, again including sex, reproduction, and *S. solidus* infection. There appears to be a complex interplay between factors, where the direction of causality in the relationship between factors determining condition, condition itself, and immune gene expression levels is an area open to further research. Condition may affect an individual's ability to respond, or response may determine condition. Furthermore, factors such as reproductive investment and *S. solidus* infection may have direct effects on immune gene expression levels while also acting indirectly through condition.

CONCLUSIONS

Studying the immune expression profiles of individuals from two wild populations of stickleback has provided new insights into the function of the immune system in a natural setting. We demonstrate that two populations differ in their immune gene expression profiles, and that a complex interplay of multiple factors correlates to individual immune expression levels within each population. There appear to be important roles of sex, reproductive status, and individual condition. Infection with a parasite with fitness consequences to the host, *Schistocephalus solidus*, relates to the innate and adaptive responses, and may represent parasite manipulation of the host. While some factors show the same pattern in the two populations studied, others only relate to gene expression levels in a single population. The present study adds to our understanding of the immune response in a natural setting, and shows that studying the effect of single factors in isolation may overlook important and complex interactions in the wild. While the patterns observed are correlative, they suggest a range of areas for further research.

ACKNOWLEDGEMENTS

We would like to thank all the members of the MacColl lab at the University of Nottingham for their assistance in sampling in the field, and Ann Lowe for her help in the laboratory. This work was funded by a Natural Environment Research Council (NERC) studentship awarded to S.R.

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